An assessment of extraction and assay techniques for quantification of calpain and calpastatin from small tissue samples[1,2]

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ABSTRACT: Our objective was to evaluate whether small (biopsy-sized) samples could be used to measure calpain and calpastatin activities in skeletal muscle. The accuracy of different separation and assay methods for the quantification of calpains and calpastatin from small (1.0 and 0.2 g) skeletal muscle samples was tested. In Exp. 1, the LM was removed from six lambs, and a 50-g subsample was processed using the reference method (DEAE-Sephacel chromatography and casein assay). Subsamples (1.0 and 0.2 g) also were processed using the two-step separation (1 mL DEAE-Sephacel and bulk elution using 200 and 400 m*M* NaCl) and heated calpastatin methods; in both cases, fractions were assayed with Bodipy-labeled and [^{14}C]-labeled casein microassays. Finally, casein zymography was used to separate and quantify the calpain proteases from 1.0- and 0.2-g samples. The values obtained after processing the 50-g sample using the reference method were judged most accurate, and the alternative approaches were compared with these. For each extraction and assay approach, we considered: 1) the effect of the sample size on the mean activity; 2) increased or decreased variation of data; and 3) the correlation relative to the reference method. Where possible, we compared the ratio of calpain to calpastatin activities determined using the alternative approaches with the ratios found using the reference method. These methodologies were further investigated in Exp. 2, where single homogenates from different tissues (heart, spleen, lung, and muscle) were assayed using the alternative approaches. Experiment 1 established that most of the approaches suffered from poor correlations and/or unacceptable variation. By using a large, homogenous sample in Exp. 2, however, we determined that this error was not due to the methodologies themselves. Therefore, the unacceptable variation found in Exp. 1 resulted from the small sample size, and we recommend that large tissue samples (e.g., 50 g) should be used for calpain and calpastatin activity measurements in skeletal muscle instead of small tissue biopsies (e.g., 0.2 and 1.0 g).

Key Words: Biopsy, Calpain, Calpastatin, Lamb, Microassay, Quantification

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Introduction

The calpain system is composed of two ubiquitous calpain proteases and their inhibitor, calpastatin. Although a definite biological function of this system remains to be established, there is strong evidence to indicate that calpains are involved in postmortem tenderization (Koohmaraie, 1992b, 1994, 1996) and muscle growth (Goll et al., 1991; Koohmaraie et al., 2002). For any investigation where sample size is limited (e.g., biopsy or small animal size), it is desirable to have accurate methodologies to separate and measure these components. The trusted, reproducible, and robust methodologies used for postmortem in vivo analysis typically rely on relatively large sample sizes (Geesink and Koohmaraie, 1999), although more sensitive assay approaches have been employed for in vitro studies of the calpain system in tissue culture (Xu and Mellgren, 2002) and myofibrils (Delgado et al., 2001). Therefore, our objective was to determine the efficacy and suitability of some of these techniques for separation and quantification of the calpain system in small tissue biopsy samples collected from a large muscle.

[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Materials and Methods

This research employed several different methodologies to separate and assay calpains and calpastatin from skeletal muscle and other tissues. Our "gradient separation" (Protocol 1) describes the processing of a 50-g tissue sample with a 212-mL DEAE Sephacel chromatography column, and is based on the protocol described by Koohmaraie (1990). Separation with a 1-mL DEAE straw-column and the "two-step separation" (Protocol 2) approach is based on the work of Geesink and Koohmaraie (1999). Our "heated calpastatin" (Protocol 3) analysis was based on the work of Shackelford et al. (1994), and the "casein zymography" (Protocol 4) was performed as described by Veiseth et al. (2001). All protocols achieved partial or complete separation of μ-calpain, m-calpain, and calpastatin, but only casein zymography (Protocol 4) has the inbuilt capacity to quantify μ-calpain and m-calpain activities. For the other separation approaches (Protocols 1, 2, and 3), an assay method is required for quantification. Our "standard assay" casein digestion, described by Koohmaraie (1990), was used to localize and quantify enzymes separated using the gradient separation (Protocol 1). Assays employing [^{14}C]-labeled casein (based on the work of Koohmaraie, 1992a) or Bodipy-labeled casein (based on the work of Thompson et al., 2000) were used to quantify calpains and calpastatin separated using two-step separation (Protocol 2) and heated calpastatin (Protocol 3).

Assessment of Assay and Extraction Techniques

The Roman L. Hruska U.S. Meat Animal Research Center Animal Care and Use Committee approved the use of animals in this study.

Experiment 1. Six Rambouillet lambs were slaughtered at 4 mo of age according to standard procedures. Within 20 min of exsanguination, approximately 100 g of LM were removed from each animal, trimmed of visible fat and connective tissue, finely diced, and mixed. From this sample, 50 g was removed for gradient separation (Protocol 1) and assayed using the standard casein assay. From the remaining 50 g, two 1-g samples and two 0.2-g samples were removed for heated calpastatin analysis (Protocol 3); one sample of each size was analyzed using [^{14}C]-labeled casein, whereas the other was assayed using Bodipy-labeled casein. The two-step separation approach (Protocol 2) was tested by extracting two 0.2-g samples and assaying fractions with either [^{14}C]-labeled or Bodipy-labeled casein. Finally, 1- and 0.2-g samples were removed for casein zymography (Protocol 4). All samples were prepared for their analysis separately.

Experiment 2. Two Rambouillet lambs were slaughtered at 6 mo of age according to standard procedures. Within 20 min of exsanguination, approximately 100 g of LM was removed from each animal, along with 100 g of lung, spleen, and heart. Tissues were trimmed of visible fat, cartilage, and connective tissue, and finely diced. A single homogenate was prepared for each tissue by homogenizing 50 g in three volumes of prerigor extraction buffer as described in the gradient separation (Protocol 1). After homogenization and centrifugation, the volume of the supernatant fraction was measured, and an aliquot equivalent to 0.5 g of tissue was removed. This aliquot was then equally divided between heated calpastatin (Protocol 3) and casein zymography (Protocol 4) separation approaches. The heated aliquot was assayed using the Bodipy-labeled casein assay. The remaining sample (equivalent to 49.5 g tissue) was dialyzed and processed according to gradient separation (Protocol 1) before being assayed using the standard casein method. In addition to assaying using the standard casein assay, pooled fractions of μ-calpain, m-calpain, and calpastatin were assayed using the [^{14}C]- and Bodipy-labeled casein assays.

Statistical Analyses. Means, SD, and simple correlations were determined using SAS (SAS Inst., Inc., Cary, NC). The CV was calculated as CV = 100 × (SD of mean/mean).

Separation Methods

For all separation experiments, tissue from muscle, heart, lung, or spleen was trimmed of visible fat, cartilage, and connective tissue before being diced into small pieces. All sample processing and manipulations were performed at 4°C unless otherwise stated.

Protocol 1: Gradient Separation. Protocol 1 was based on the approach described by Koohmaraie (1990), with some modifications. Briefly, extracts were prepared by homogenizing 50 g of tissue in three volumes of prerigor extraction buffer (50 m*M* Tris base, 10 m*M* EDTA, 0.05% 2-mercaptoethanol [**MCE**], 2 m*M* phenylmethylsulfonyl fluoride [**PMSF**], 100 mg/L of ovomucoid, and 16 mg/L of leupeptin, adjusted with HCl to pH 8.3). Homogenization was performed using a Waring blender (Dynamics Co. of America, New Hartford, CT) three times for 30 s on high speed, interspersed with 30-s cooling periods. The homogenate was centrifuged at 16,000 × *g* (maximum force) for 2 h, and the supernatant fraction was dialyzed against dialysis buffer (40 m*M* Tris base, 5 m*M* EDTA, and 0.05% MCE, pH 7.35) overnight. The following day, samples were clarified by centrifugation at 28,000 × *g* (maximum force) for 1 h and filtered over glass wool before loading by gravity onto 212-mL DEAE-Sephacel columns equilibrated with elution buffer (40 m*M* Tris base, 0.5 m*M* EDTA, and 0.05% MCE, pH 7.35). Columns were washed with elution buffer until the absorbance at 278 nm of the outflow was less than 0.1. Bound proteins were eluted with a linear gradient from 25 to 500 m*M* NaCl at 40 mL/h, and 5-mL fractions (n = 140) were collected. The proteolytic activities of μ-calpain and m-calpain, and the inhibitory activity of calpastatin in these fractions were determined using a standard casein assay.

Protocol 2: Two-Step Separation. Protocol 2 was based on the approach described by Geesink and Koohmaraie (1999), with some modifications. Briefly, an extract was prepared by homogenizing 0.2 g of tissue in five volumes of prerigor extraction buffer, using a fine-tip probe powered by a Polytron homogenizer (Brinkman Instruments, Westbury, NY) set on speed setting 6 (medium speed). Three 15-s homogenization bursts were interspersed with 15-s cooling periods. The homogenate was centrifuged at 10,000 × *g* (maximum force) for 30 min at 4°C. To decrease sample conductivity, the volume of the supernatant fraction was increased to 10 mL with elution buffer, and the diluted sample was gravity loaded onto a 1-mL DEAE Sephacel straw column that had previously been washed extensively with elution buffer. To coelute calpastatin and μ-calpain, elution buffer containing 200 m*M* NaCl was applied to the column, and fractions were collected. To elute m-calpain, elution buffer containing 400 m*M* NaCl was used. The proteolytic activities of μ- and m-calpain, and the inhibitory activity of calpastatin, were determined using both Bodipy- and [^{14}C]-labeled casein. The coelution of μ-calpain and calpastatin demanded that half of each 200 m*M* NaCl elution fraction be heated at 95°C for 15 min, cooled on ice for 15 min, and then centrifuged for 15 min at 8,800 × *g* (maximum force). The heated supernatant fraction was then removed and assayed for calpastatin using semipurified μ-calpain as a control. The activity of μ-calpain was estimated by subtracting the calpastatin activity in the 200 m*M* NaCl fractions before and after heating. Fractions containing calpain or calpastatin activity were pooled and the combined volume was reassayed.

Protocol 3: Heated Calpastatin. Protocol 3 was based on the approach described by Shackelford et al. (1994), with some modifications. Briefly, an extract was prepared by homogenizing either 1 or 0.2 g of tissue in six volumes of prerigor extraction buffer as described for the two-step separation (Protocol 2). The homogenate was then centrifuged at 10,000 × *g* (maximum force) for 30 min at 4°C. One-milliliter aliquots of the supernatant fraction were transferred into 2-mL microfuge tubes, heated at 95°C for 15 min, and cooled on ice for 15 min before being centrifuged at 8,800 × *g* (maximum force). Supernatant fractions were then collected and, where appropriate, aliquots from the same original sample were recombined. Samples were assayed for calpastatin using Bodipy-labeled and [^{14}C]-labeled casein.

Protocol 4: Casein Zymography. Protocol 4 was based on the approach described by Veiseth et al. (2001), with some modifications. Briefly, an extract was prepared by homogenizing either 1 or 0.2 g of tissue in six volumes of prerigor extraction buffer as described for the two-step separation (Protocol 2). The homogenate was then centrifuged at 10,000 × *g* (maximum force) for 30 min at 4°C, before removing an aliquot of the supernatant fraction for casein zymography. Due to differences (anticipated from unpublished data from our laboratory) in the quantity of μ-calpain and m-calpain from different tissues, a variety of sample volumes was loaded onto the casein gels. For extracts from skeletal muscle, aliquots equivalent to 4 mg of tissue were loaded for the detection of both μ- and m-calpain. For lung, spleen, and heart, the volumes were 2 and 0.4 mg, 1 and 1 mg, and 10 and 3 mg for detection of μ- and m-calpain, respectively. These amounts were calculated based on reported activity levels from these tissues, and were aimed to ensure that proteolytic activities would not exceed the sensitivity of the zymogram based on the results of Veiseth et al. (2001). The aliquot was processed and zymography was performed exactly as described by Veiseth et al. (2001).

Assay Methods

Standard Assay. The standard assay followed the protocol described by Koohmaraie (1990), and it was used exclusively for determination of calpain and calpastatin activity resulting from gradient separation (Protocol 1). Fractions were individually assayed to localize calpain and calpastatin activity, and then pooled and reassayed in triplicate to accurately determine the units of activity per gram of tissue.

Bodipy-Labeled Casein Assay. The Bodipy assay is based on the method of Thompson et al. (2000) with some modifications. Briefly, a range of sample volumes (5 to 25 μL) were combined with sample dilution buffer (20 m*M* Tris base, 1 m*M* EDTA, 100 m*M* KCl, and 0.1% MCE, pH 7.5) in black microtiter plates to a final volume of 100 μL. The reaction was initiated by adding 100 μL of Bodipy casein (5 μg of Bodipy-labeled casein/mL) in sample dilution buffer containing 6 m*M* $CaCl_2$. The microtiter plate was covered with foil and incubated at 37°C for 30 min before fluorescence was measured using 485-nm excitation and 535-nm emission in a Wallac Victor2 1420 Multilabel Counter (Perkin Elmer Life Sciences, Downers Grove, IL). All assays were performed in quadruplicate. For quantification of calpastatin, semipurified ovine m-calpain was introduced to the sample before the addition of sample dilution buffer to a final volume of 100 μL.

[^{14}C]-Labeled Casein Assay. Fractions of heated supernatant were assayed for calpain or calpastatin activity using [^{14}C]-labeled casein as described by Koohmaraie (1992a). Briefly, for calpain, [^{14}C]-labeled casein was incubated with a range of sample dilutions in a 100-mL reaction (10 m*M* Tris base and 10 m*M* $CaCl_2$, pH 7.5). The reaction was stopped after incubation for 1 h at 25°C by the addition of 100 μL of cold 10 mg/mL BSA and 200 μL of cold 10% trichloroacetic acid. Undegraded casein was separated by centrifugation at 8,800 × *g* (maximum force) for 15 min at 4°C, and 200 μL of the trichloroacetic acid-soluble proteins were mixed with 5 mL of scintillation fluid (ScintiVerse, Fisher Scientific, Pittsburgh, PA). Radioactivity of the mixture was measured using a Packard TriCarb 1600TR liquid scintillation analyzer (Packard Instruments, Meriden, CT). For

quantification of calpastatin, a volume of semipurified ovine lung m-calpain was included in the incubation reaction.

Results and Discussion

Our overall goal in this study was to establish whether biopsies would permit an accurate and repeatable quantification of calpain and calpastatin activities in skeletal muscle. The combination of the gradient separation (Protocol 1) and the standard casein assay is the typical separation/assay approach used in this laboratory for the quantification of calpain and calpastatin. In our experiment, this robust combination was reliable and accurate, and provided reproducible data. Therefore, calpain and calpastatin activities determined by gradient separation and the standard assay were assumed to be accurate and were used as the benchmark for all other approaches. For clarity, this combination will be referred to as the "reference method."

Experiment 1

For assays using Bodipy-labeled and [^{14}C]-labeled casein, raw data were collected as fluorescence units or counts per min, respectively. To make comparisons easier, these units were converted to the standard assay units per gram of tissue (**STD units**) as defined by Koohmaraie (1990). This conversion was made by assaying a volume of DEAE-Sephacel purified m-calpain using all three assay approaches (standard assay, Bodipy, and [^{14}C]casein) and calculating the appropriate conversion factor. Activities determined for μ-calpain, m-calpain, and calpastatin using the reference method were comparable with previously reported values for ovine LM (Koohmaraie 1990; Koohmaraie et al., 1991), and serve to underscore the reliability of this quantification approach.

As shown in Table 1, the calculated mean STD units for samples extracted using heated calpastatin (Protocol 3) were consistent using either assay method and ranged from 3.97 to 4.11 STD units. These values were all slightly higher than the 3.62 STD units determined by the reference method; however, this disparity was not unexpected because the heated calpastatin separation approach is known to overestimate calpastatin activity (Shackelford et al., 1994). The CV value is a normalized SD (expressed as a percentage of the mean), and provides a measure of how widely the sample values spread from the mean. Coefficient of variation percentages for heated calpastatin STD Units were relatively consistent and varied from 14.9 to 18.8%, whereas the CV of the reference method was 19.1%. Based on these results, it would seem that, relative to the reference method, the heated calpastatin approach (Protocol 3) provides an accurate measurement of calpastatin using both Bodipy-labeled and [^{14}C]-labeled casein. This encouraging result was reassessed, however, due to the erratic correlation values (r), which ranged from 0.27 to 0.91. These correlations showed that samples with relatively high calpastatin (determined using the reference method) were not consistently identified as "high," nor were low calpastatin samples consistently identified as "low." A notable exception to this was the 0.2-g heated calpastatin extraction followed by [^{14}C]-labeled casein, which displayed the highest (r = 0.91) correlation with the reference method.

The failure to correlate the activities of the same samples using two different extraction and assay approaches was repeated in the two-step extraction approach (Protocol 2). In Table 1, correlations between the reference method and the two-step separation range from 0.56 to 0.75 for μ-calpain, m-calpain, and calpastatin assayed with Bodipy and [^{14}C]-labeled casein. Of more concern, however, was the fact that the calculated STD units underestimated the actual activity (determined from the reference method) for both calpains and calpastatin, and that the CV values ranged from 20.1 to 56.4%. Thus, for 0.2-g samples, the two-step separation coupled with a Bodipy- or [^{14}C]-labeled casein assay was an inaccurate method that underestimated enzyme activity and introduced variability.

The final separation technique, zymography (Protocol 4), incorporates the assay approach within its methodology. After capturing a digital image of the stained zymogram, it is possible to quantify protease activity of both μ- and m-calpain, relative to an internal control using densitometry software. For the purpose of our investigations, one animal was chosen as the control, and a sample from this animal was included on all zymograms. Sample size did not seem to influence the mean activity of either μ- or m-calpain. The average activity for μ-calpain was 64 and 62% for 1- and 0.2-g samples respectively, whereas the activity for m-calpain was 31 and 32% for 1- and 0.2-g samples, respectively. This uniformity is promising, but the relative difference between the calpains is inaccurate. According to zymograms, there is an approximate 2:1 ratio of μ-calpain to m-calpain, whereas results from the reference method establish this ratio to be 1.2:1, which is more typical of postmortem lamb. In addition, the CV values ranged from 15.6 to 49.7%, and the correlations ranged from 0.23 to 0.50. Overall, these results demonstrate that zymography was unaffected by sample size; however, it exaggerated the difference between μ- and m-calpain and was inaccurate.

None of the separation and assay approaches used in Exp. 1 emerged as a promising microassay candidate. Cumulatively, these observations raise the possibility that 1) variations in calpain and calpastatin exist within the LM and these variations are accentuated when removing small samples; 2) sample preparation (e.g., homogenization) is inconsistent when preparing small samples; and 3) the separation and assay methodologies are insensitive and inaccurate. Therefore, our

Table 1. The quantification of calpain and calpastatin from ovine LM (fresh-tissue basis) using Protocols 1 through 4 and comparison with the reference method (Exp. 1)

Separation assay:	Gradient standard[a]	Heated calpastatin[b]				Two-step separation[c]		Zymography densitometry[d,f]	
		Bodipy[e]		[^{14}C]-Casein[e]		Bodipy[e]	[^{14}C]-Casein[e]		
Sample size:	50 g	1 g	0.2 g	1 g	0.2 g	0.2 g	0.2 g	1 g	0.2 g
Item									
Calpastatin mean	3.62	3.97	4.06	3.99	4.11	1.55	1.72	—	—
SD	0.7	0.7	0.6	0.6	0.7	0.4	0.5	—	—
CV, %	19.1	18.8	14.7	14.9	16.6	24.0	27.5	—	—
Correlation(r)[g]	—	0.27	0.76	0.62	0.91	0.74	0.58	—	—
μ-Calpain mean	1.52	—	—	—	—	0.68	0.50	64	62
SD	0.18	—	—	—	—	0.1	0.3	16.5	9.6
CV, %	10.9	—	—	—	—	20.1	56.4	25.8	15.6
Correlation(r)[g]	—	—	—	—	—	0.56	0.67	0.23	0.50
m-Calpain mean	1.27	—	—	—	—	0.88	0.64	31	32
SD	0.14	—	—	—	—	0.3	0.2	15.2	10.1
CV, %	12.2	—	—	—	—	30.0	30.9	49.7	31.4
Correlation(r)[g]	—	—	—	—	—	0.74	0.75	0.28	0.34

[a,b,c,d]Protocols 1, 3, 2, and 4, respectively.
[e]Original units from Bodipy and [^{14}C]-casein assays were converted to standard units/g.
[f]Percent activity relative to control.
[g]Correlation with values from reference method analysis of 50-g sample.

objective in Exp. 2 was to investigate the last of these three possibilities.

Experiment 2

Previously, assays of heart, lung, spleen, and LM have shown that these tissues contain widely different activities of μ-calpain, m-calpain (unpublished data), and calpastatin (Koohmaraie et al., 1995; Lorenzen et al., 2000). These tissues were therefore chosen to provide samples with varying calpain and calpastatin activities. The objective in preparing a uniform homogenate was to assess the efficacy of the separation techniques; however, separating the same sample using different approaches will produce the same activities only if the assay methods are accurate. To address this second point, the pooled fractions collected and assayed in the reference method also were assayed using the [^{14}C]-labeled casein and Bodipy-labeled casein approaches. The fluorescence units and counts per minute produced from Bodipy-labeled and [^{14}C]-labeled casein assays were not adjusted to produce calculated STD units as they were in Exp. 1.

As presented in Table 2, the correlations between activities of pooled fractions determined from these assay methods were convincing. For example, m-calpain values from both microassay approaches were strongly correlated (r = 0.99) with the reference method. Although this initially led us to consider that these assay methodologies were highly accurate, the broad range of values were a direct consequence of the tissue selection, and when combined with a small number of samples (n = 8), it was not unexpected that the correlation coefficients would be so high. If the spread of the data is considered, the methods seem less attractive. Determined by the reference method, the least and greatest μ-calpain activities (irrespective of tissue) were 0.48 and 3.95 STD units, respectively, which was approximately an eightfold difference. For m-calpain, activities ranged over a ninefold difference, whereas calpastatin produced a 15-fold difference. When assaying the same pooled samples with [^{14}C]-labeled casein, μ-calpain and m-calpain displayed a 12- and 94-fold range, respectively, between the least and greatest reported values. Using Bodipy-labeled casein, the ranges for μ-calpain, m-calpain, and calpastatin were 5-, 13-, and 15-fold respectively. Thus, the high correlations demonstrated that the assays were reliably able to rank greater and lesser activities, but contrasting the spread of data generated by the [^{14}C]-labeled and Bodipy-labeled casein assays indicates these methods were inaccurate relative to the standard assay.

Combining the heated calpastatin (Protocol 3) separation with Bodipy assay produced a range of values that correlated well with both the reference method (r = 0.97) and Bodipy-assayed pooled calpastatin (r = 0.91; data not shown). Moreover, both μ-calpain and m-calpain activities measured using zymography (Protocol 4) showed good correlations with the reference method (Table 2). These approaches independently used aliquots of the homogenized sample to determine whether the separation approach influenced the activity measurements. Because the methodologies seem capable of reliably ranking activities, and the ranking was consistent between independently separated samples, we can conclude that these separation methods are effective and do not introduce excessive error into the correlation.

Overall Discussion

The results obtained in Exp. 1 showed that none of the separation and assay approaches gave accurate and

Table 2. The quantification of calpain and calpastatin from ovine LM, heart (HRT), spleen (SPL), and lung (LNG) on a fresh-tissue basis collected from two lambs (1 and 2; Exp. 2)

	Pooled fractions from Reference Method DEAE separation									
	Regular assay[a]			[^{14}C]-Casein[b]		Bodipy-casein[c]		Heated Bodipy[c]	Zymography[d]	
Sample	μ-Calpain	m-Calpain	Calpastatin	μ-Calpain	m-Calpain	μ-Calpain	m-Calpain	Calpastatin	Calpastatin	μ-Calpain	m-Calpain
LM 1	1.40	1.91	3.69	1,882	5,823	4,420	2,374	3,797	3,216	23.31	23.89
LM 2	1.39	1.38	2.63	1,863	4,399	4,737	1,705	2609	2,444	23.18	19.99
HRT 1	0.72	2.85	39.71	840	32,224	4,303	5,007	39,779	23,731	6.50	32.56
HRT 2	0.48	2.05	20.71	618	22,454	2,468	3,185	14,741	17,629	7.58	27.22
SPL 1	3.30	4.89	6.13	6,967	137,978	7,732	6,153	5,342	5,972	79.15	78.20
SPL 2	3.95	3.56	6.54	7,255	104,589	13,277	6,309	5,643	6,140	82.83	80.55
LNG 1	2.83	11.97	15.63	4,599	415,061	11,228	21,601	11,898	14,104	41.08	213.13
LNG 2	3.31	12.99	12.41	4,369	374,799	7,816	19,906	9,273	10,907	43.92	215.13
Correlation(r)[e]				0.96	0.99	0.89	0.99	0.98	0.97	0.93	0.99

[a]Standard units/g.
[b]Counts per min/mg of tissue.
[c]Fluorescent units/mg of tissue.
[d]Percent activity relative to control.
[e]Correlation with values from reference method analysis of a 50-g sample.

reliable results from biopsies of ovine LM. In Exp. 2, we observed that the assay methodologies correlated well with the standard assay, and that the separation approaches (Protocols 3 and 4) did not introduce error; however, quantification of enzyme activities seems to be particular to the separation and assay method used. For example, the heated extraction procedure overestimates calpastatin activity, and [^{14}C]-labeled casein is more sensitive to elevated levels of m-calpain than Bodipy-labeled casein when both are compared with the reference method. Nevertheless, values obtained using the microassay techniques were reliable to rank samples of greater and lesser activities.

Although the reference method was found to be very reliable, showing little assay-to-assay variation, both the Bodipy-labeled casein assay and zymography were susceptible to this type of variation. Specifically, we found that identical samples assayed at successive times do not produce the same data value (data not shown), although the trends within an assay were consistent. This assay-to-assay variation can, however, be monitored and corrected by introducing a standard sample that is included in all assays (Veiseth et al., 2001).

Results of Exp. 2 demonstrated that when a homogenous sample from a large tissue sample (50 g) was separated and assayed using different approaches, high correlations between reference method and alternative methods were possible. Therefore, the poor correlations noted in Exp. 1 were most likely a result of inconsistent sample processing or variation in enzyme activity between different locations within the muscle. It is reasonable to suggest that small tissue samples may be susceptible to variation from a location effect; however, successful biopsy sampling has been reported previously. Using 5-g samples, Wheeler and Koohmaraie (1991) surveyed the LM and found that calpain and calpastatin activities did not vary among different regions. More investigation is required to determine whether significant variation exists within the LM and, if so, to determine what sample size is convenient for a biopsy procedure, while remaining representative of the whole muscle. Consequently, at this time, we do not recommend the use of biopsies (e.g., 0.2 to 1.0 g) for quantification of calpain and calpastatin activities in skeletal muscle. For reliable and accurate quantification, the best approach is a large sample size (relative to muscle size) combined with the reference method.

Implications

Results of this study imply that biopsies should not be used for quantification of calpain and calpastatin activities in skeletal muscle. At this time, we recommend that large sample sizes, together with the standard separation and assay technique, be used when determining calpain and calpastatin activities from skeletal muscle. Biopsies do not provide reliable absolute measures of activity, but can, under certain condi-

tions, be used to rank samples within an experiment. A significant barrier to achieving accurate quantification from small tissue samples may be localized variations in enzyme activity and/or inconsistent extractions. The collection of biopsy samples should be preceded by experiments assessing the significance of tissue variation and the development of certifiable extraction and homogenization procedures.

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